

AMENDMENTS TO THE SPECIFICATION

Please amend the paragraph beginning on page 6, line 14 by inserting a close parenthesis as follows:

Figure 1 shows a gene organization of *BpmI* restriction-modification system. Genes *BpmIRM* and *BpmIM1* code for *BpmI* endonuclease (*BpmI* endonuclease-methylase fusion protein) and *BpmI M1*, respectively. *BpmI*-Δ#1, *BpmI*-Δ#2, and *BpmI*-Δ#3 are deletion mutants with deletions in the methylation or specificity domains.

Please add new Figures 9 and 10-1, 10-2, 10-3 and 10-4 and insert two new paragraphs beginning on page 8, line 16 prior to the heading **DETAILED DESCRIPTION OF THE EMBODIMENTS** to describe new Figures 9 and 10-1, 10-2, 10-3 and 10-4 as follows:

Figure 9 shows the sequence alignment of Gamma motifs from alignment of N6mA DNA Mtases and 9N4mC DNA Mtases as described in Figure 1c of Malone et al. J. Mol. Biol. 253:618-632 (1995).

Figures 10-1 – 10-4 show the amino acid sequences of BpmI, AcuI, BsgI and ThaIV. Conserved motifs have been annotated.

Please amend the paragraph beginning on page 10, line 16 by substituting the number in "Example 3" to "Example 4" as follows:

In one approach to the above problem, a nucleic acid linker is inserted into DNA encoding the methylase or specificity domain of one Type IIG restriction endonuclease. The linker may be sufficient in length to encode 3-12 amino acids. A DNA encoding a complementary portion of a second Type IIG restriction endonuclease or a portion of a complementary region of a second Type IIG restriction endonuclease or all or part of an independent γ -type methylase (not derived from a Type IIG endonuclease) but containing a specificity region which is ligated to the linker. The chimeric DNA encodes a functional restriction endonuclease with altered specificity. Example 3 4 describes how a DNA linker coding for up to about 10 amino acids may be inserted between the coding region for a methylase and the coding region for the restriction endonuclease (in this example, *BpmI*) such that a second methylase region and a specificity region is added to the linker.

Please amend the paragraph beginning on page 30, line 16 by substituting the number in "Figure 2" to "Figure 6" as follows:

The N-terminal 300 bp coding sequence was amplified in PCR reaction 1 with the following PCR conditions: 94°C for 5 min, 1 cycle; 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec for 20 cycles; 72°C, 7 min for 1 cycle, 4 units of Vent® DNA polymerase. The rest of the coding sequence was amplified in PCR reaction 2 with the following PCR condition: 94°C for 5 min, 1 cycle; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min for 20 cycles; 72°C, 7 min for 1 cycle, 4 units of Vent® DNA

polymerase. PCR products 1 and 2 were purified from a low-melting agarose gel and used as the template for PCR assembly using primers P1 and P4. The assembly PCR conditions were 94°C for 5 min, 1 cycle; 94°C for 30 sec, 55°C for 30 sec, 72°C for 3 min for 20 cycles; 72°C, 7 min for 1 cycle. The mutagenized PCR product was purified and digested with *Xba*I and *Bam*HI and cloned into a T7 expression vector pET28a. The phenotype of the resulting *Bpm*I variant should be R⁻ (cleavage deficient) and M⁺ (methylation proficient). After screening 18 plasmids for PCR insert and digestion with *Bpm*I endonuclease, 11 clones were found to be resistant to *Bpm*I digestion (data shown in Figure 26). Endonuclease activity was not detected in any of the mutant extracts prepared from IPTG-induced cells. Because multiple rounds of PCR were performed to generate the R⁻M⁺ variant D74A, it was necessary to re-sequence the entire gene to confirm that no other mutations were introduced. Six sequencing primers were used to sequence the entire gene. R⁻M⁺ variant D74A clone #4 carried one additional amino acid change at E1007G. In a separate experiment, it was determined that E1007G substitution was not important to *Bpm*I endonuclease activity. The Asp74 to Ala74 substitution abolished *Bpm*I endonuclease activity.

Please amend the paragraph beginning on page 32, line 7 by replacing "g" with "gamma" as follows:

The methylase domains of *Bpm*I and *Acu*I belong to the gamma type N6 adenine methylases. Motif IV is a conserved methylase block and has a GNPPY sequence in both of the *Bpm*I and *Acu*I methylase domains. This site was chosen as a fusion

junction for making chimeric enzymes. An *AcuI* deletion mutant was constructed that deleted methylase motif IV and the remaining C-terminal coding sequence. The starting *AcuI* enzyme was a cleavage-deficient variant D80A (R-M+ mutant). The codon Phe520 was mutated to a stop codon by PCR mutagenesis to generate variant *AcuI* D80A/Δ(520-1000). The deletion mutant protein was expressed in *E. coli* ER2566 via T7 expression vector pET28a. When the cells were induced with IPTG (3 hours induction at 37°C), a prominent protein band of 59 kDa was detected in SDS-PAGE gel (data shown in Figure 7). The deletion mutant *AcuI* D80A/Δ (520-1000) is soluble in *E. coli* cell extract and not degraded by *E. coli* proteases. This deletion mutant can be used as the backbone to construct chimeric Type IIG enzymes. DNA coding for similar methylase motifs IV to VIII and an alternate specificity determinant can be ligated to this deletion mutant to construct a functional chimeric enzyme.

Please amend the paragraph beginning on page 34, line 19 by adding motif "X" and substituting motif "VIII" for "X" as follows:

The DNA recognition sequences for *BpmI* and *BsgI* are CTGGAG and GTGCAG, respectively. *BsgI* endonuclease is a Type IIG enzyme that shares 35.4% amino acid sequence identity to *BpmI*. A chimeric enzyme was constructed between *BpmI* and *BsgI*, in which the N-terminal coding sequence (catalytic domain plus methylase motifs X, I to III) was derived from *BpmI* and the C-terminal coding sequence (methylase motifs IV to VIII and the specificity domain) was derived from *BsgI*. The chimeric coding sequence was generated by a two-step PCR reaction. PCR

primers were designed that can anneal to methylase motif IV on both *BpmI* and *BsgI* templates. The amino acid sequences in the fusion junction are shown below:

BpmI F D A I I G N P P Y

BsgI F D V I L G N P P Y

The forward primer P1 described in Example 5 and a new reverse primer P2' were used to amplify the N-terminal coding sequence from *BpmIRM* gene.

The new reverse mutagenic primer P2' has the following sequence:

5' ATAGGGTGGATTGCCTAATATTACATCAAAGCCACCATTGCGC 3'
(P2'). (SEQ ID NO:25)

PCR conditions were 94°C for 5 min, 1 cycle; 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min for 17-22 cycles; 72°C, 7 min for 1 cycle with 4 units of Vent® DNA polymerase.

The forward mutagenic primer in the fusion junction has the following sequence:

5' TTTGATGTAATATTAGGCAATCCACCCCTATATAAGAATTC 3' (P3')
(SEQ ID NO:26)

Since the *BsgIRM* gene was cloned in pUC19, primer P3' and a pUC universal primer NEB #1221 was used to amplify the C-terminal *BsgI* coding sequence. PCR conditions were 94°C for 5 min, 1 cycle; 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min for 15-22 cycles; 72°C, 7 min for 1 cycle with 4 units of Vent® DNA polymerase. The PCR products were purified from a low-melting agarose gel and assembled by PCR using primers P1' and pUC universal primer #1221 (New England Biolabs, Inc., Beverly, MA). The PCR conditions were 94°C for 5 min, 1 cycle; 94°C for 30 sec, 55°C for 30 sec, 72°C for 3 min 10 sec for 15 cycles; 72°C, 7 min for 1 cycle with 4 units of Vent® DNA polymerase.

The PCR DNA fragment was cloned into pET21at and transformed into T7 expression host ER2566. *E. coli* host with pACYC-BpmIM or pACYC-BsgIM was also used for transformation. The fusion junction was confirmed by DNA sequencing.

Please amend the paragraph beginning on page 36, line 8 by substituting the number in "Figure 4" to "Figure 8" as follows:

E. coli strain ER1992 carries the *dinD::lacZ* fusion (the *dinD* DNA damage inducible promoter is fused to the *lacZ* gene). When bacterial DNA is damaged by double-stranded cuts or single-stranded nicks, UV radiation, or interference with DNA replication, the indicator strain forms blue colony on X-gal plates. When plasmids carrying the chimeric *BpmI/BsgI* R-M fusion were transformed into the endo-blue indicator strain ER1992 (*dinD::lacZ*), they caused formation of blue colonies in the absence of IPTG induction. This indicates transformants suffered DNA damage resulting from constitutive expression of the fusion protein (data shown in Figure 48). The transformants initially formed blue colonies on X-gal plates. When these cells were plated on X-gal plates with IPTG, most cells turn white. After IPTG induction, cells suffered lethal level of DNA damage and died. The cells carrying inactive mutant version of the chimeric R-M fusion protein took over the population and formed the white colonies. When the blue transformants were re-streaked on X-gal plates, about two-third remains blue colonies, and one-third form white or partial blue colonies.